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Distinct spatiotemporal roles of hedgehog signalling during chick and mouse cranial base and axial skeleton development

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ABSTRACT

The cranial base exerts a supportive role for the brain and includes the occipital, sphenoid and ethmoid bones that arise from cartilaginous precursors in the early embryo. As the occipital bone and the posterior part of the sphenoid are mesoderm derivatives that arise in close proximity to the notochord and floor plate, it has been assumed that their development, like the axial skeleton, is dependent on Sonic hedgehog (Shh) and modulation of bone morphogenetic protein (Bmp) signalling. Here we examined the development of the cranial base in chick and mouse embryos to compare the molecular signals that are required for chondrogenic induction in the trunk and head. We found that Shh signalling is required but the molecular network controlling cranial base development is distinct from that in the trunk. In the absence of Shh, the presumptive cranial base did not undergo chondrogenic commitment as determined by the loss of *Sox9* expression and there was a decrease in cell survival. In contrast, induction of the otic capsule occurred normally demonstrating that induction of the cranial base is uncoupled from formation of the sensory capsules. Lastly, we found that the early cranial mesoderm is refractory to Shh signalling, likely accounting for why development of the cranial base occurs after the axial skeleton. Our data reveal that cranial and axial skeletal induction is controlled by conserved, yet spatiotemporally distinct mechanisms that co-ordinate development of the cranial base with that of the cranial musculature and the pharyngeal arches.

Introduction

The cranial base, a supportive and protective structure underlying the brain of gnathostomes, arises from multiple cartilaginous condensations which expand and fuse to form a united structure running from the foramen magnum posteriorly to the interorbital junction anteriorly. At the midline three bones, the occipital, sphenoid and ethmoid, are formed which extend laterally to unite with other cartilaginous elements including the developing nasal, otic and optic capsules (Vorster, 1989; McBratney-Owen et al., 2008). The cranial base is formed from both neural crest and mesoderm (Couly et al., 1993; McBratney-Owen et al., 2008). Fate mapping studies in chicks and mice have shown that the structures that are formed anterior to the notochord (*i.e.* prechordal cranium) are derived from neural crest whilst, the post-chordal (posterior) cranial base is derived from the mesoderm (Couly et al., 1993; Evans and Noden, 2006; McBratney-Owen et al., 2008; Wada et al., 2011). In both mice and chicks, the prechordal:postchordal interface lies within the basisphenoid bone and the ethmoid bone is entirely neural crest derived, whilst the occipital bone is derived from somitic and unsegmented cranial mesoderm (Couly et al., 1993; McBratney-Owen et al., 2008).

The post-chordal or posterior cranial base develops in close proximity to the notochord and ventral neural tube and it is assumed that these tissues induce its formation in a fashion analogous to the induction of the axial skeleton. In the trunk, formation of the sclerotome, the progenitor of the vertebrae, requires Hedgehog signalling from the notochord, floor plate and endoderm, together with Noggin/Gremlin antagonism of Bmp signalling (Christ et al., 2004; Dockter, 2000; Fan and Tessier-Lavigne, 1994; McMahon et al., 1998; Monsoro-Burq, 2005; Stafford et al., 2011; Zhang et al., 2001). Previous studies have suggested some degree of similarity between trunk and cranial base skeletal development. In mice and zebrafish, Shh and the chondrogenic marker Sox9 are expressed in adjacent tissues and loss of Shh signalling following cyclopamine treatment or genetic inactivation of components of the Shh signalling pathway affect the development of the cranial base (Jeong et al. 2004; Nie et al., 2005; Eberhart et al., 2006; Schwend and Ahlgren, 2009; Wada

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et al., 2005). Interestingly, the anterior elements, *i.e.* the neural crest derived trabecular cartilages which give rise to the ethmoid bone, appear to be the most susceptible (Wada et al., 2005; Eberhart et al., 2006; Koyama et al., 2007; Aoto et al., 2009; Schwend and Ahlgren, 2009). The timing of when Shh signalling is required in cranial base development, together with its specific role, is unknown.

It is also known that head and trunk mesoderm display different histological and molecular characteristics expressing distinct sets of genes (Bothe and Dietrich, 2006; Hacker and Guthrie, 1998). This suggests that the response of the head mesoderm to specific signals might be different from that of the trunk. This has been clearly highlighted by comparison of axial *versus* craniofacial myogenesis: in the head, Wnt signalling acts to prohibit myogenic commitment in contrast to the trunk where Wnt signalling is required for this process (reviewed by Bryson-Richardson and Currie, 2008; Noden and Francis-West, 2006).

Here, we have examined the development of the cranial base with the aim of understanding the signals that control its formation and how its development compares temporally to the musculoskeletal system of the trunk and pharyngeal arches. The head:trunk divide falls at the occipito-cervical boundary interface and therefore, the cranial base includes the occipital bone, sphenoid and ethmoid bones. Specifically we compared the development of the cranial base to that of the trunk axial skeleton in chick and mouse embryos. We found that, as for myogenesis, formation of the cranial skeleton occurs later than the trunk skeleton and involves a unique molecular repertoire. However, we show the role of Shh signalling is conserved during vertebrae and cranial base development and that posterior cranial base development occurs later than the axial skeleton because the early cranial mesoderm is refractory to Shh signalling. We propose this delay is necessary to allow expansion of the brain and also to co-ordinate development of the cranial base with that of the musculoskeletal system of the pharyngeal arches.

Materials and methods

Chick and mouse embryos

Fertilised White Leghorn chicken eggs (Henry Steward & Co. Ltd, Lincolnshire, UK) were incubated at 38 ± 1 °C and staged as described by Hamburger and Hamilton (1951). Wild type and *Shh*$^{-/-}$ E11.5 and E12.5 mouse embryos were generated as described (Chiang et al., 1996). Embryos were fixed in 4% paraformaldehyde and were either analyzed by *in situ* hybridization for gene expression, histological staining or immunolabeling.

In situ hybridisation to whole mounts and tissue sections

Embryos were processed into methanol for whole mount *in situ* hybridisation or into wax for *in situ* hybridisation to tissue sections. Whole-mount *in situ* hybridisation using digoxigenin-labelled RNA probes and *in situ* hybridisation to tissue sections using ^{35}S-labelled RNA riboprobes were performed as described by (Dastjerdi et al., 2007). Chick probes were synthesised as described previously: *cShh* (Johnson et al., 1994), *cPtc1* and *cPtc2* (Pearse et al., 2001), *cSox9* and *cCollagen type II* (Healy et al., 1999), *cGli1* and *cGli3* (Marigo et al., 1996), *cGli2* (Schweitzer et al., 2000), *cPax1* (Muller et al., 1996), *cBapx1* (Rodrigo et al., 2003), *cIhh* (Vortkamp et al., 1996), *cCollagen type X* (Oshima et al., 1989), *cBmp2* and *cBmp4* (Francis-West et al., 1994). Mouse probes were synthesised as described previously: *mSox9* and *mCollagen II* (Zhao et al., 1997), *Ptc1* (Goodrich et al., 1996).

Embryonic manipulations

Affi-Gel Blue agarose beads (BioRad) were incubated with 1 mg/ml of recombinant murine Noggin, Dickkopf 1, or Sfrp2 (R&D Systems) for 1 h at 37 °C and were applied into the cranial mesoderm adjacent to the mesencephalon of HH stage 10 chick embryos (Supplementary Fig. 1A). Trunk notochords isolated from the level of somites 5–10 (1 being the most rostral somite) of HH stage 10 chick embryos were transplanted into the cranial paraxial mesoderm adjacent to the mesencephalon of HH stage 10 chick embryos (Supplementary Fig. 1B). Rostral notochord from the level of the mesencephalon was also transplanted ectopically in the cranial mesoderm as above (Supplementary Fig. 1B) or into the trunk between the neural tube and the somite in the prospective lumbar region. Stage HH13/14 chick embryos were treated with cyclopamine (1 mg/ml cyclopamine (Sigma) in 45% solution of 2-hydroxypropyl-β-cyclodextrin (HBC) in PBS) *in ovo* according to (Cordero et al., 2004). A 45% solution of 2-hydroxypropyl-β-cyclodextrin in PBS was used as a control of HBC toxicity. The embryos were allowed to develop for 24 h and fixed for *in situ* hybridization and immunolocalisation analyses to whole mounts or tissue sections.

Immunohistological studies

Immunohistological studies were carried out on paraffin fixed tissues following standard protocols. Sections were dewaxed in xylene for 30 min and rehydrated through a decreasing ethanol series into ddH_2O for 5 min each. Antigen retrieval was carried out by boiling the slides in 10 mM sodium citrate buffer pH 9.0 for 10 min. Antibodies (Rabbit polyclonal phospho-Smad 1/5/8 (Cell Signalling Technology), phospho-Histone H3 (NEB), cleaved Caspase 3 (NEB)) were used at a 1:100 dilution. Sections were incubated with the primary antibody overnight at 4 °C. Subsequently, the sections were rinsed three times in 1xPBS for 5 min each. Secondary antibody solution (Alexa Fluor 488 (Invitrogen) diluted at 1:1000 concentration) was applied for 1 h at room temperature and the sections were washed as previously. Sections were mounted in ProLong Gold Antifade mounting medium (Invitrogen).

Histological analysis

Tissue sections were stained with Haematoxylin and Eosin or Alcian Blue 8GX according to standard protocols.

Results

Cranial base and trunk have distinct temporal developmental patterns and molecular signatures

To compare the initial stages of cranial base and axial skeleton development, we carried out whole-mount *in situ* expression analyses of key molecular signals known to be essential for sclerotome development, including the homeobox-containing genes *Pax1* and *Bapx1* and the chondrogenic markers *Sox9*, *type II collagen*, *Sox5* and *Sox6* in HH stage 10 chick embryos. We focused on these genes because *Pax1* induces the expression of *Bapx1* in the sclerotome (Rodrigo et al., 2003) that, in turn, induces and maintains the expression of *Sox9* (Murtaugh et al., 2001; Zeng et al., 2002). Interestingly, we found that *Bapx1* was expressed in the unsegmented mesoderm of the developing cranial base in the absence of *Pax1* expression (Fig. 1A and B) and that *Pax1* expression was restricted to the pharyngeal endoderm (white arrow in Fig. 1A′). In addition, the cranial

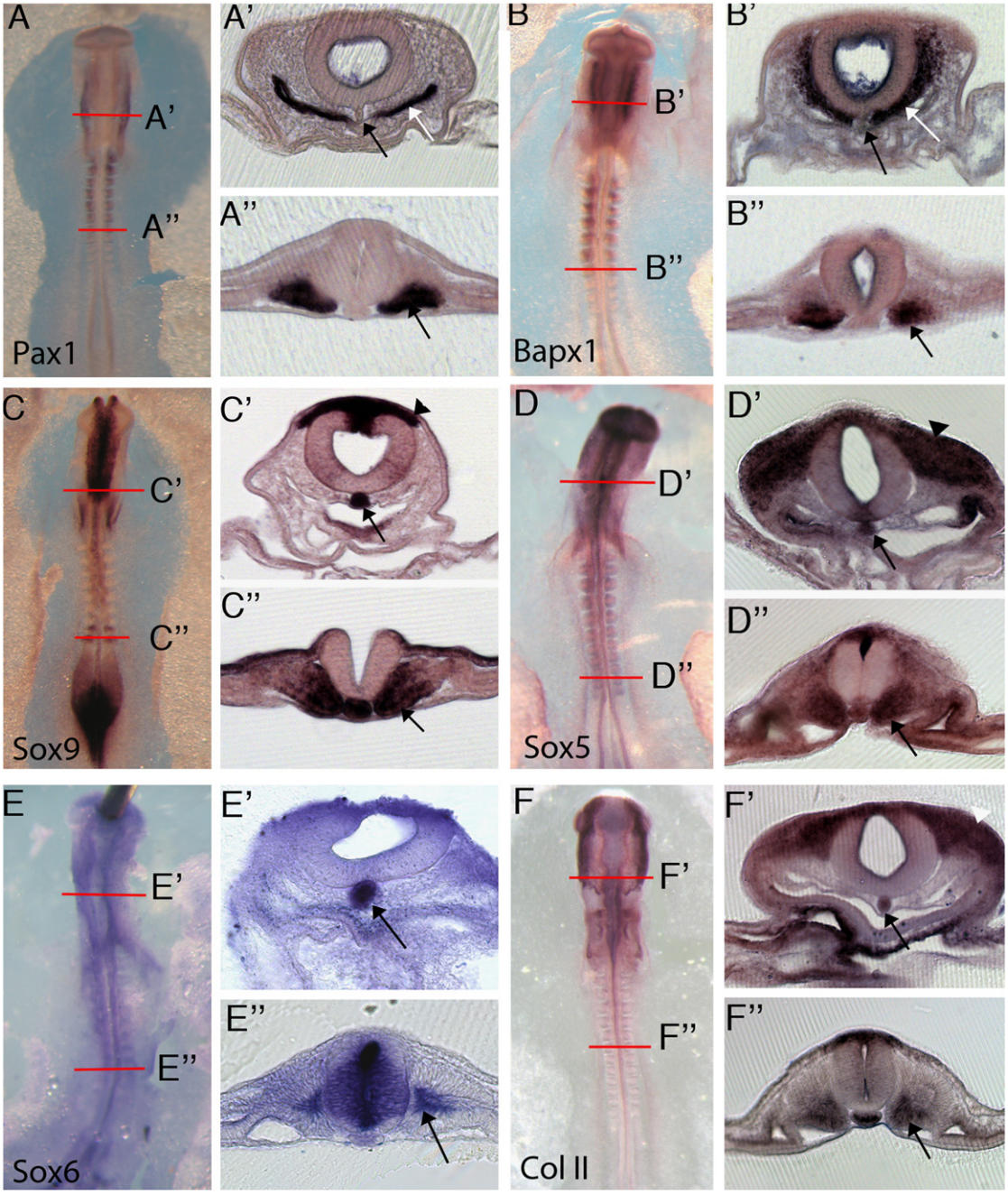

Fig. 1. Chondrogenic commitment occurs later in the developing cranial base relative to the trunk axial skeleton. Whole-mount *in situ* hybridisation analysis showing the expression of *Pax1* (A), *Bapx1* (B), *Sox9* (C), *Sox5* (D), *Sox6* (E) and *type II collagen* (F) in HH stage 10-11 chick embryos. A–F are dorsal views of the embryo, A′–F′ and A″–F″ are transverse sections at the levels indicated in A–F through the cranial unsegmented mesoderm and trunk paraxial mesoderm respectively. The notochord is arrowed in A′–F′ whilst the sclerotome is arrowed in A″–F″. White arrows in A′ and B′ indicate the cranial endoderm and mesoderm, respectively. Arrowheads in C′, D′ and F′ indicate cranial neural crest cells.

mesoderm displayed minimal if any expression of *Sox9*, *Sox6*, *Sox5* and *type II collagen* at this stage although these factors were expressed in the migrating cranial neural crest cells (Fig. 1C–F; arrowhead in C′, D′ and F′). In contrast, *Pax1* and *Bapx1* were co-expressed in the developing sclerotome that, as expected, displayed also very strong expression of *Sox9*, *Sox5*, *Sox6* and *type II collagen* (Fig. 1C–F; C″–F″). The *Sox5, Sox6, Sox9* and *type II collagen* genes were expressed in the notochord at every anatomical location examined (Fig. 1, black arrows in C′–F′). Clearly the cranial mesoderm displays a unique molecular profile and a delay in chondrogenesis when compared to trunk mesoderm.

Histological and molecular analyses of cranial base development

We then investigated later stages of development by histological and molecular analyses to determine when the cranial base starts to form and the timing and rate of differentiation. Embryos ranging from HH stage 18 to HH stage 36, when terminal differentiation has occurred in the axial and appendicular skeleton, were first analysed histologically by H&E and alcian blue staining. In chicks, several embryonic cartilages form at the midline which include the parachordal cartilage at the level of the mesencephalon, the acrochordal cartilage at the tip of the

notochord, and the trabaecular cartilages, which arise anterior to the notochord (Romanoff, 1960). Caudal to the parachordal cartilage, the occipital somites also condense to contribute to the basi-occipital and exo-occipital cartilages (Romanoff, 1960; Couly et al., 1993).

At HH stage 18 the developing cranial base largely consisted of disorganised loose mesenchyme surrounding the notochord (Fig. 2A). The first morphological signs of cranial base became apparent by HH stage 21 when a region of higher cellular density—likely representing the developing acrochordal cartilage—was present at the rostral tip of the notochord and adjacent to Rathke's pouch (arrowheads in Fig. 2B). At this stage the parachordal cartilages, which develop adjacent to the notochord, were not appreciable morphologically (Fig. 2B). By HH stage 24, the acrochordal, parachordal and optic cartilages were readily visualised by H&E and alcian blue staining, the latter indicative of the production of cartilaginous matrix (Fig. 2C, D and E; Fig. 3M; data not shown). The posterior region of the parachordal cartilage, which contributes to the basisphenoid bone, encompassed the notochord (arrowed in Fig. 2D) and was not yet fused with the developing basioccipital cartilages derived from the paraxial mesoderm of the occipital somites. The boundary is marked by the arrowheads in Fig. 2D. However, rostrally the parachordal cartilages were now fused with the developing acrochordal cartilages, which had also started to expand across the medial–lateral axis (Fig. 2E; data not shown). By HH stage 27, the trabeculae cartilages, representing the most anterior cartilages of the cranial base, had formed (Fig. 2F; Supplementary Fig. 2C; Wada et al., 2011). The occipital cartilages derived from the occipital somites were still distinct from the developing parachordal cartilage at HH stage 27, but were fused by HH stage 30, uniting the cranial base along the anterior-posterior axis (Supplementary Fig. 2D and E). By HH stage 36, the acrochordal and parachordal cartilages increased in size across the medio-lateral axis to unite with the optic and otic capsules, respectively (Supplementary Fig. 2L; data not shown). At this stage the chondrocytes were morphologically immature and there was no obvious sign of pre-hypertrophy or hypertrophy.

Tissue sections from representative stages and locations were processed for *in situ* hybridisation analysis. *Bapx1* expression was

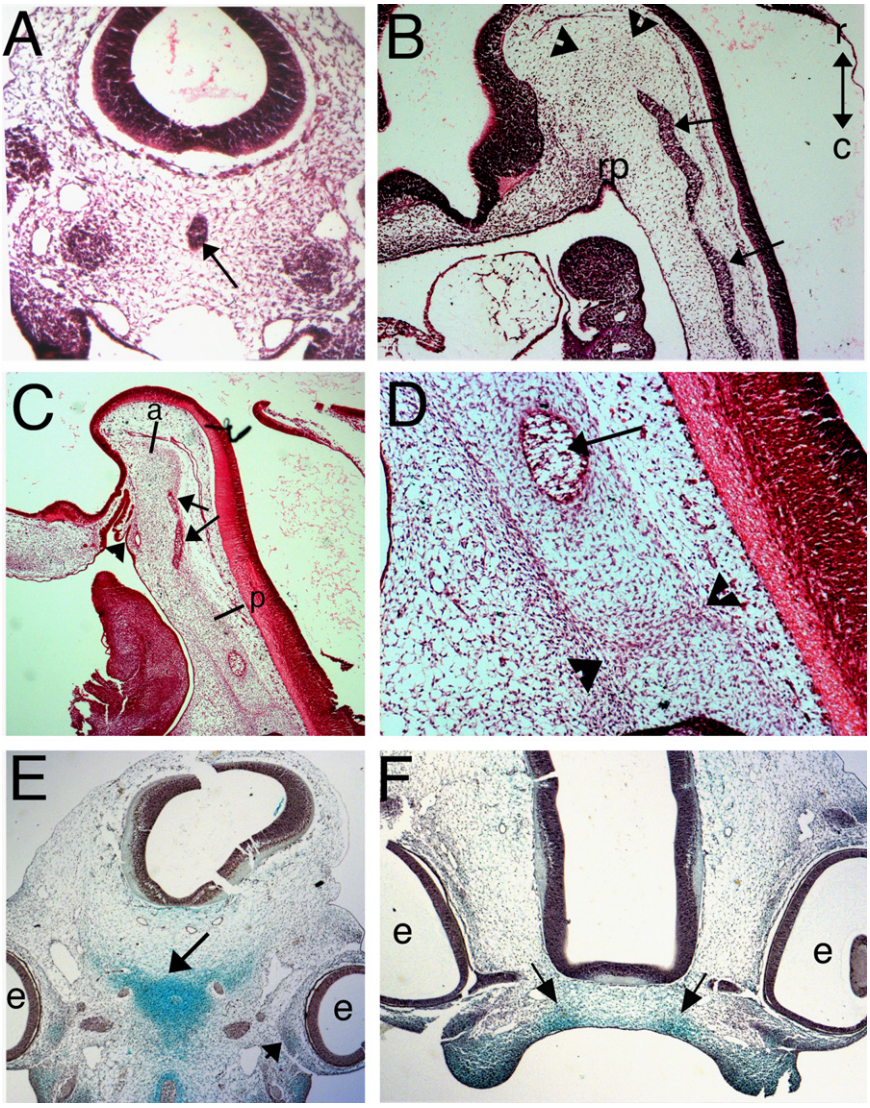

Fig. 2. Histological analysis of the development of the cranial base. Frontal (A, E and F) and saggital sections (B, C and D) through the developing cranial base of HH stage 18 (A), 21 (B), 25 (C, D and E) and 27 (F) chick embryos stained with haemotoxylin and eosin (A–D) or alcian blue (E and F). (D) shows a higher power image of the parachordal and occipital cartilages in (C). The notochord is arrowed in (A–D). The arrows in (E) and (F) indicate the parachordal and trabecular cartilages respectively. The arrowheads demarcate the acrochordal cartilage in (B), Rathke's pouch in (C), the parachordal:occipital cartilage boundary in (D) and the developing optic capsules in (E). a, acrochordal cartilage; e, eye; p, parachordal cartilages; and rp, Rathke's pouch.

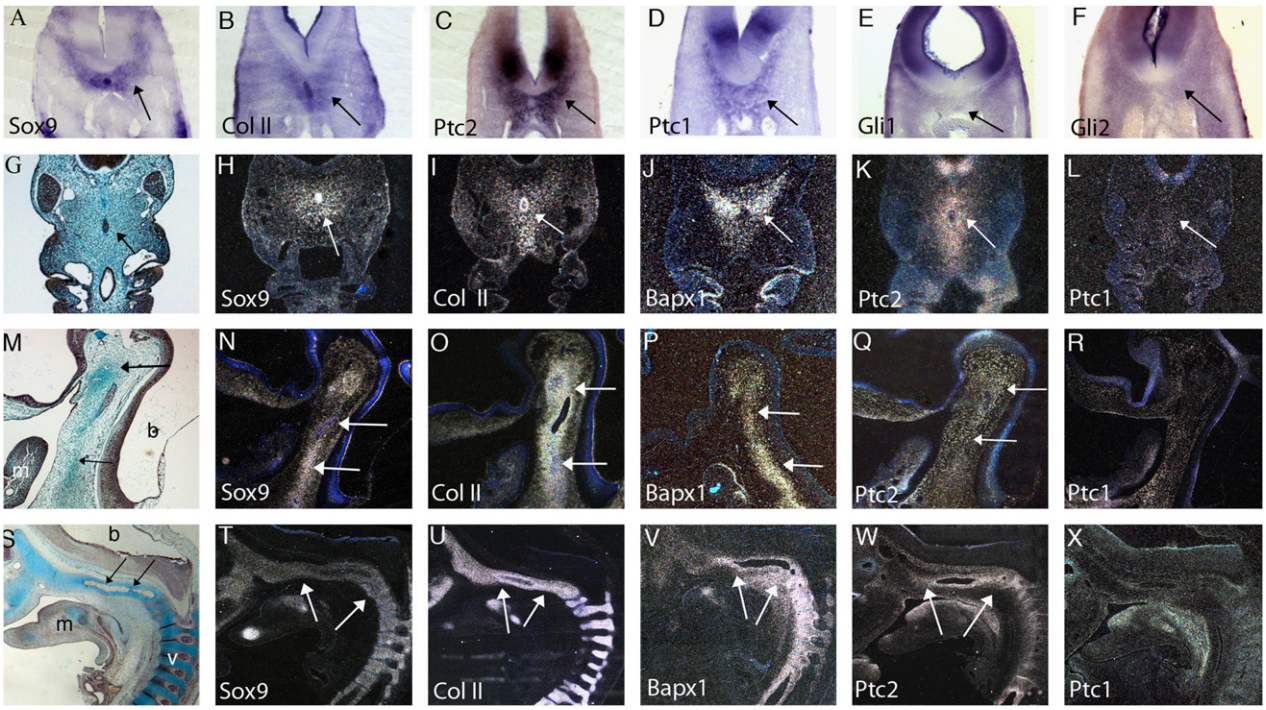

Fig. 3. Chondrogenic commitment in the developing cranial base coincides with the activation of Shh signalling. (A–F) show vibratome sections of chick embryos following whole-mount DIG *in situ* hybridisation. (G, M and S) show alcian blue stained tissue sections, together with adjacent tissue sections (H, I, K and L; N–R and T–X respectively) probed for gene expression by radioactive *in situ* hybridisation. The sections show the expression of *Sox9* (A, H, N and T), *type II collagen* (B, I, O and U), *Ptc2* (C, K, Q and W), *Ptc1* (D, L, R and X), *Gli1* (E), *Gli2* (F) and *Bapx1* (J, P and V), in the developing cranial base of stage 15 (C–F), 17 (A), 18 (B, G, H, I, K and L), 19 (J), 24 (M–R) and 30 (S–X) chick embryos. A–L are transverse sections through the developing cranial base whilst (M–X) are sagittal sections. A–X, the arrows indicate the developing cranial base. In M, the upper arrow indicates the acrochordal cartilage and the lower arrow, the parachordal cartilage. B, brain; M, mandibular primordia; and v, vertebrae

consistently observed in both the acrochordal and parachordal cartilages from HH stage 18 to stage 30 (Fig., 3J and P). Its expression appeared slightly lower in the acrochordal cartilage by HH stage 30 and was also down-regulated along the midline of the cranial base (Fig. 3V; data not shown). Expression of the chondrogenic genes *Sox9* and *type II collagen* was apparent at every stage studied (Fig. 3A, B, H, I, N, O, T and U; Supplementary Fig. 2F, G, M and N). Interestingly, *Ihh*, a marker of prehypertophic chondrocytes, was not detected until HH stage 36 (Supplementary Fig. 2H and O) and *type X collagen*, a marker of terminal chondrocyte maturation, was still not expressed (Supplementary Fig. 2I). This is in dramatic contrast to the axial and appendicular skeleton where *Ihh* and *type X collagen* expression are activated within 72 h of the initial chondrogenic commitment (data not shown) and in contrast to the pharyngeal arch cartilages that had reached hypertrophy by HH stage 36 (Supplementary Fig. 2I). Therefore, in addition to the later onset of chondrogenic induction, the developing cranial base is also characterized by a delay in cartilage maturation and hypertrophy, analogous to the difference in the rate of myogenic differentiation between the head and trunk reported in previous studies (Noden and Francis-West, 2006; Noden et al., 1999). Although the parachordal cartilages are thought to arise as two separate condensations that subsequently fuse along the midline, we found no molecular or histological evidence for any distinct condensations along the medial-lateral axis. Similarly, McBratney-Owen et al., 2008 did not observe two separate condensations during their analysis of cranial base development in mice and they suggested that the two independent "entities" may fuse at the prechondrogenic stages. Therefore, differentiation of the parachordal cartilages appears to occur directly around the notochord, which is a process distinct from chondrogenic induction in the trunk which occurs at a distance from the notochord. In the trunk committed sclerotomal cells then migrate towards and surround the notochord. In contrast to the acrochordal and parachordal cartilages, the trabecular cartilages did clearly develop as two separate entities (Fig. 2F; also see Wada et al., 2011).

Activation of shh signalling coincides with chondrogenic commitment within the cranial mesoderm

Despite the molecular/temporal differences with trunk mesoderm, the posterior cranial base arises in close proximity to the notochord, and we hypothesized that Shh signalling would still be required for its development analogous to Shh roles in trunk chondrogenesis. To address this possibility, we examined expression of the Hh receptors *Ptc1* and *Ptc2* and down-stream effectors *Gli1*, *-2* and *-3*. *Ptc1*, *-2* and *Gli1* are transcriptional targets of the Shh signalling pathway and therefore, their expression is indicative of Shh signalling activity (Goodrich et al., 1996; Pearse et al., 2001).

At HH stage 15, *Ptc1*, *Ptc2*, *Gli1* and *Gli2* transcripts were clearly detected in the cranial paraxial mesoderm (Fig. 3C–F) and thus, their expression precedes the onset of *Sox9* and *collagen type II* expression which occurs at HH stage 17 (Fig. 3A and B) supporting the possibility that Shh signalling plays a role in the development of the posterior cranial base (Nie et al., 2005; Koyama et al., 2007). Expression of *Ptc2*, in association with *Sox9* and *type II collagen* expression, continued throughout the developing cranial base until at least HH stage 30 (Fig. 3K, Q and W). In contrast, *Ptc1* expression was down-regulated and negligible between HH stages 20 to 30 (Fig. 3L, R and X). At HH stage 36, *Ptc1* and *Ptc2* were both expressed in the perichondrial cells of the cranial base (Supplementary Fig. 2J, K, P and Q), and *Ptc2* was also expressed in the chondrocytes adjacent to the notochord (Supplementary Fig. 2Q). Curiously, activation of Shh signalling did not coincide with chondrogenic commitment within the otic and optic capsule (Supplementary Fig. 2A and B; data not shown).

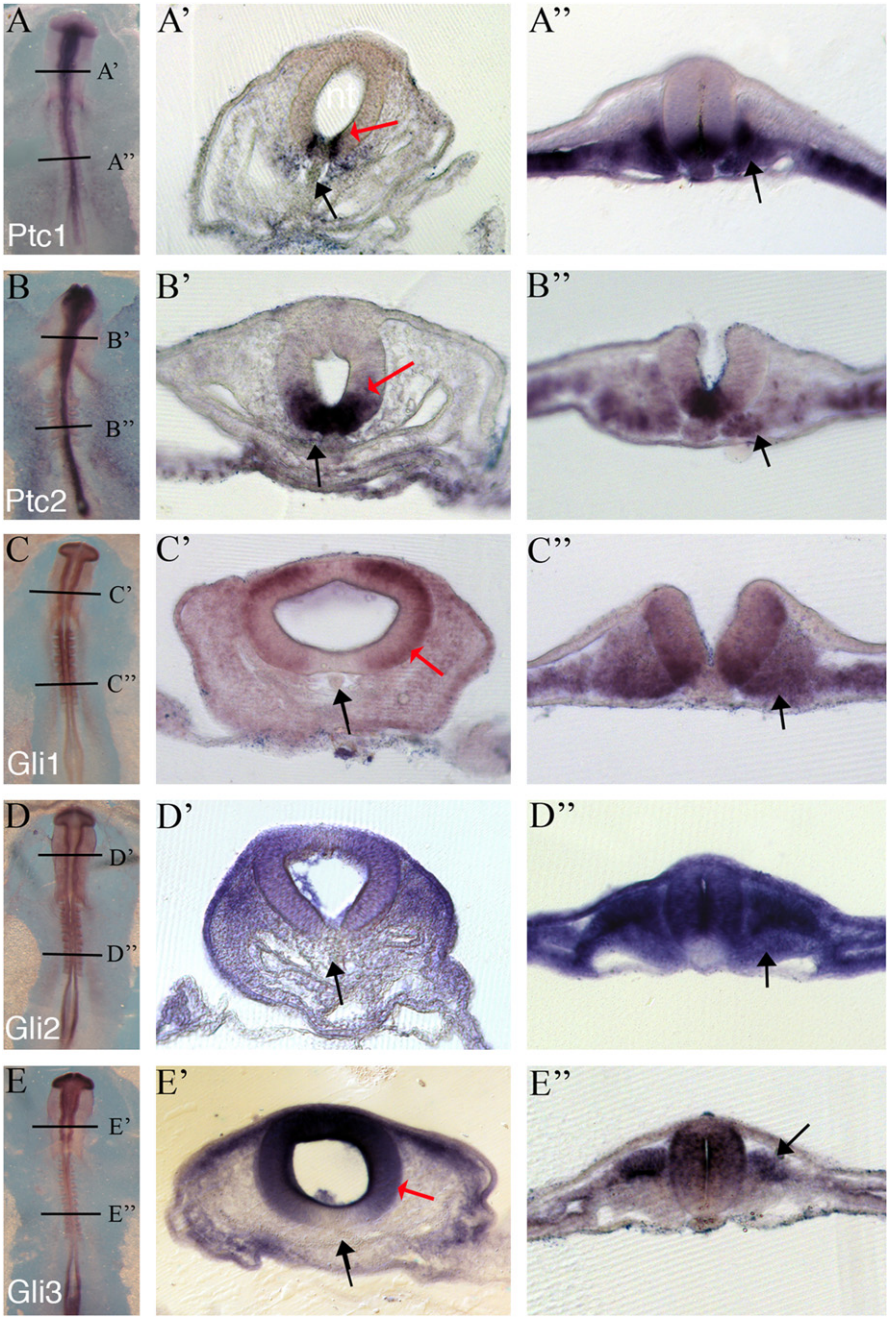

Fig. 4. The Shh signalling pathway is not active in the early cranial mesoderm. DIG—*in situ* hybridisation showing the expression of *Ptc1* (A), *Ptc2* (B), *Gli1* (C), *Gli2* (D) and *Gli3* (E) in HH stage 10 chick embryos. A–E are dorsal views of whole embryos and A′–E′ and A″–E″ are vibratome sections at the level indicated. The black arrows in A′–E′ indicate the cranial notochord, which is in the centre of the presumptive cranial base, whilst the red arrows indicate expression in the neural tube. The arrows in A″–D″ indicate the developing sclerotome. The arrow in E″ indicates the dermomyotome.

To further ascertain whether Shh signalling activity may coincide with, and be required for, early cranial base development, we analysed younger embryos. At HH stage 10, *Ptc1* and *Ptc2* expression was already robust in the sclerotome (Fig. 4A″ and B″) and cranial ventral neural tube (Fig. 4A′ and B′), but expression in the cranial mesoderm was very weak and limited to a small number of cells immediately adjacent to the notochord and the floor plate (Fig. 4A′ and B′). *Gli1* transcripts were clearly detected in developing sclerotome and cranial neural tube, but were not detectable in the cranial paraxial mesoderm at HH stage 11 (Fig. 4C′ and C″). In the trunk, high levels of *Gli2* and *Gli3* expression were detected in the dorsal somite (Fig. 4D″ and E″) and in the head their expression was detected within the neural tube and migrating neural crest cells (Fig. 4D′ and E′). Therefore, there was no indication of Shh signalling activity within the presumptive cranial base at these stages. Since *Shh* is expressed throughout the anterior-posterior axis of the notochord and the floor plate of the neural tube (Supplementary Fig. 3A–A″) the combined data suggest that in contrast to the trunk paraxial mesoderm, the cranial mesoderm is not competent to respond to Shh signalling at HH stage 10-11 but becomes competent to respond by HH stage 15. Thus, the cranial mesoderm becomes responsive to Hh signalling over developmental time and this onset of competence just precedes induction of the cranial base.

To investigate if the cranial base also develops later than the trunk axial skeleton in mammals and if chondrogenic commitment

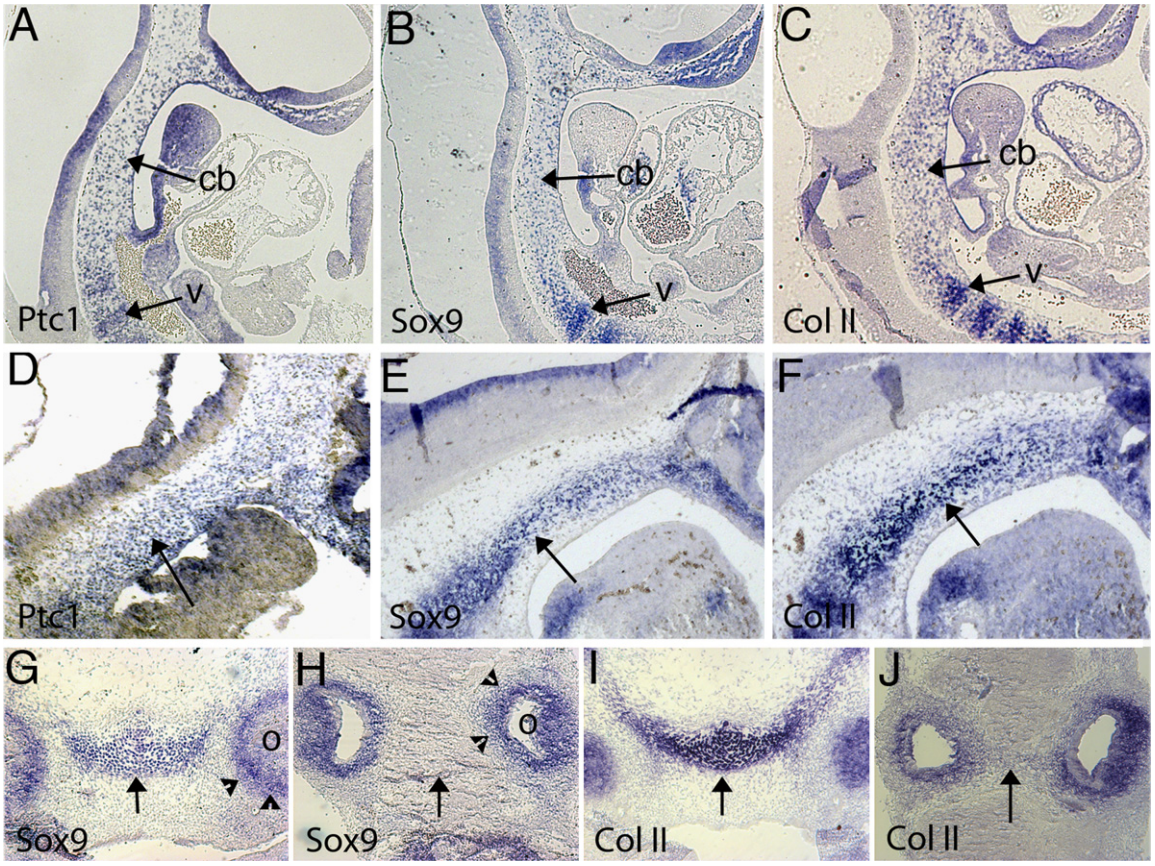

Fig. 5. Shh signalling and development of the mammalian cranial base. DIG—*in situ* hybridisations to saggital (A–F) and frontal (G–J) tissue sections through E10.5 (A–C), E11.5 (D–F) and E12.5 (G–J) wildtype (A–F, G and I) and *Shh*$^{-/-}$ mutant (H and J) mouse embryos probed for the expression of *Ptc1* (A and D), *Sox9* (B, E, G and H), and *type II collagen* (C, F, I and J). Arrows in (D–J) indicate the cranial base and arrowheads in (G and H) point to the otic capsule condensations. cb, developing cranial base; o, otic capsule; and v, vertebrae.

coincides with activation of Shh signalling, we carried out *in situ* analysis of *Sox9*, *type II collagen* and *Ptc1* expression in developing mouse embryos. We found that *Ptc1*, *Sox9* and *type II collagen* are not detectable in the cranial base at E10.5, although they are expressed in the developing sclerotome (Fig. 5A–C; data not shown). *Ptc1* expression was first detectable in the presumptive cranial base at E11.5 (Fig. 5D), when *Sox9* and *type II collagen* expression was also first apparent (Fig. 5E and F, also see Nie et al., 2005; McBratney-Owen et al., 2008). This contrasts dramatically with the trunk where *Sox9* expression was readily detectable within the sclerotome at E9.5 (Zhao et al., 1997; data not shown). Thus, in the mouse the onset of chondrogenesis in the cranial base also occurs later than in the trunk and coincides with responsiveness to Shh signalling (Fig. 5D and E).

Shh is required for cranial base development

Because chondrogenic differentiation in cranial mesoderm defined by *Sox9* and *type II collagen* expression coincides with activation of Shh signalling, it suggests that, as for the axial trunk skeleton, Shh signalling is actually required for chondrogenic development (Chiang et al., 1996; Zhang et al., 2001; Stafford et al., 2011). To investigate this possibility, we first examined cranial base development in E11.5 and E12.5 *Shh*$^{-/-}$ mutant mice. At these stages in wild type embryos, the cartilage condensations of the cranial base and early chondrogenesis are apparent, respectively (Fig. 5G and I; data not shown). DIG *in situ* hybridisation analysis revealed that the expression of *Sox9* and *type II collagen* in the presumptive cranial base was negligible/absent in *Shh*$^{-/-}$ embryos at both E11.5 and E12.5 (E11.5, $n=2$; E12.5, $n=3$; Fig. 5H and J; data not shown), although expression of both *Sox9* and *type II collagen* was detectable in the developing neural tube and otic capsule (compare Fig. 5G and I with Fig. 5H and J; data not shown). No hypertrophic chondrocytes were observed, and cells in the presumptive cranial base were all mesenchymal/fibroblastic in appearance ruling out the possibility that in the absence of Shh signalling chondrocyte differentiation had been accelerated (data not shown).

As the notochord degenerates in *Shh*$^{-/-}$ mutants (Chiang et al., 1996), the absence of cranial base development may be due to a loss of notochordal factors other that Shh. It may also reflect requirements for Shh earlier in development. Therefore, we also examined the role of Hh signalling as the cranial base is forming using the chick embryo as a model system when we can specifically inactivate Shh signalling just prior to the development of the cranial base. In this case, HH stage 13 chick embryos were exposed to cyclopamine, an antagonist of Hedgehog signalling, and were analysed at stage HH 18/19. Control embryos were treated with hydroxy-β-cyclodextrin. The treated embryos were analysed for the expression of *Ptc1*, *Ptc2* and/or *Gli1* by DIG *in situ* hybridization to tissue sections. Embryos showing negligible expression of these transcriptional targets were analysed for the expression of chondrogenic markers on adjacent tissue sections (Fig. 6E).

Treated embryos appeared narrower along the medial-lateral axis compared to control treated embryos, consistent with the role of Shh signalling during expansion of the midline (compare Fig.6A–D with Fig. 6E–H). Expression of *Sox9* ($n=4/4$), *type II collagen* ($n=3/3$) and *Sox5* ($n=2/2$) was negligible in the presumptive cranial base (Fig. 6F–H), although *type II collagen* was expressed in the notochord and cranial ectoderm indicating the

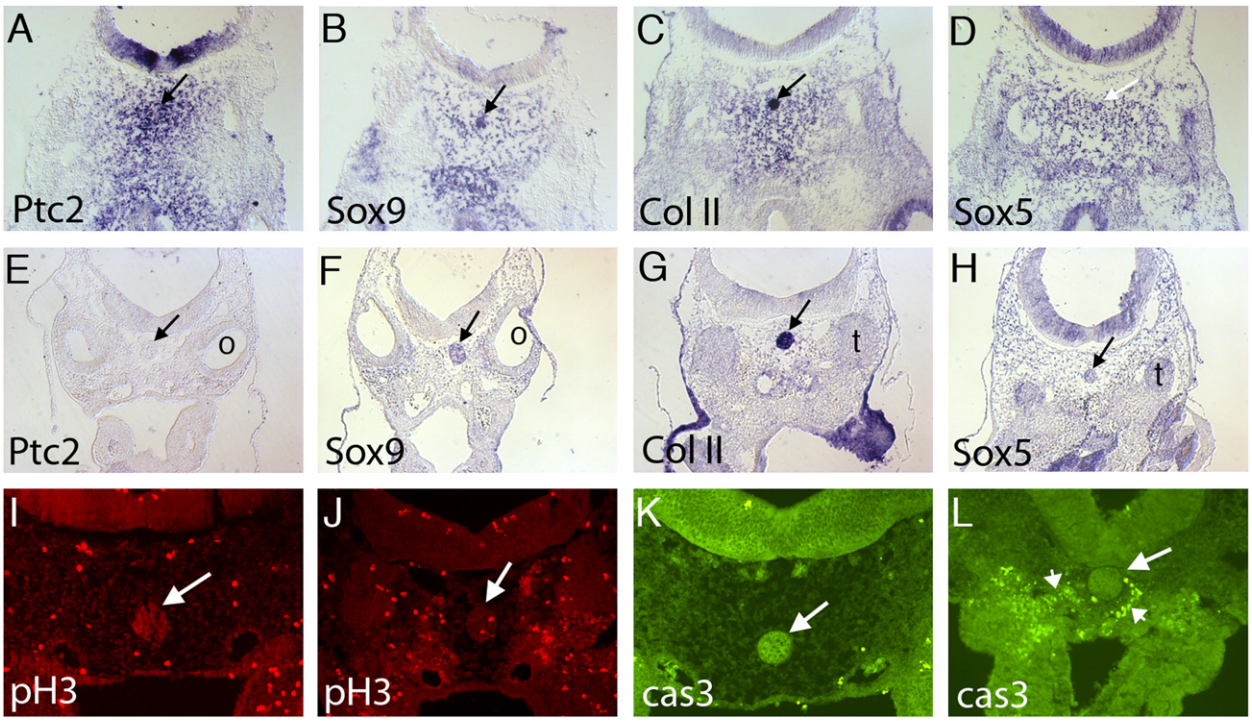

Fig. 6. Shh signalling is required for cranial base development in the chick. Transverse sections through HH stage 18 chick embryos that have been treated with cyclopamine (E–H, J and L) or the control solution (A–D, I and K) showing the expression of *Ptc2* (A, E), *Sox9* (B, F), *type II collagen* (C,G) and *Sox5* (D,H). Sections (I–L) have been immunostained with antibodies against phospho Histone H3 (I and J) or activated Caspase 3 (K and L) to identify proliferating and apoptotic cells respectively. The long arrows indicate the notochord; the short arrows in L indicate apoptotic cells clustered around the notochord. O, otic vesicle; and t, trigeminal ganglion.

tissue specific down-regulation of *type II collagen* in the presumptive cranial base (Fig. 6G). In contrast, control treated embryos showed robust expression of these markers in the developing cranial base (*Sox9* (n=3/3), *type II collagen* (n=3/3) and *Sox5* (n=2/2)) (Fig. 6A–D). As *Sox9* and *type II collagen* are specifically expressed during early chondrogenesis, we also considered the possibility that *Sox9* and *type II collagen* down-regulation resulted from the premature differentiation of the cranial base in the absence of Hh signalling. However, we did not observe the presence of differentiated chondrocytes in H&E stained cyclopamine-treated embryos (data not shown). Thus loss of Hh signalling impedes the initiation of chondrogenesis in the cranial base of both mouse and chick embryos.

Loss of HH signalling results in increased cell death in the cranial base

To assess whether changes in cell survival and/or proliferation could contribute to the failure of chondrogenic commitment during interference with Hh signalling, cyclopamine and control treated chick embryos were immunostained for activated Caspase 3 and phospho Histone H3 respectively. Clusters of apoptotic cells were present around the notochord and in developing pharyngeal arches in cyclopamine treated, but not in control, embryos (Fig. 6K and L, $n=3$). Cell proliferation was slightly, but significantly increased, in the developing cranial base of cyclopamine treated embryos compared to the controls (Fig. 6I and J, $n=3$). Shh has been shown to promote cell cycle progression at the G1 phase and the G2/M transition and the increase in the numbers of cells positive for phospho Histone H3, which marks the G2/M phase, may reflect delayed cell cycle progression in the absence of Shh. Alternatively, as chondrogenic commitment would normally result in a decrease in cell proliferation, this increase may also be a secondary consequence of the inhibition of chondrogenic commitment in the cranial base of cyclopamine treated embryos.

Why does the cranial base develop later than the trunk axial skeleton?

Given that Shh is expressed throughout the anterior-posterior axis of the notochord and neural tube (Supplementary Fig. 3), it was curious that cranial base development occurs later than the trunk skeleton. We considered three possible explanations. First, there may be antagonists of Shh signalling in the early cranial mesoderm and surrounding tissues that inhibit Shh function. Second, there may be qualitative/quantitative differences in the ability of the notochord/floor plate to signal, as suggested by a study examining the notochordal regulation of *Pax1* expression (Muller et al., 1996). Third, the distinct cranial molecular code may be prohibitive for Shh signalling. To test these possibilities, we carried out the following two sets of experiments.

Several studies have shown that Bmp action and canonical Wnt signalling can antagonize Shh signalling including during sclerotome induction (Stafford et al., 2011). Thus, we analysed *Bmp-2*, *-4* and *-7* expression and activity during early cranial chick development. We found that *Bmp7* is expressed in the floor plate throughout the developing midbrain and hindbrain *i.e.* adjacent to the developing posterior cranial base at HH stage 10 (Fig. 7C; data not shown). *Bmp2* is weakly expressed in the floor plate (Fig. 7A). In contrast, *Bmp4* is expressed in the neural folds and neural crest and is not in close proximity to the developing cranial base (Fig. 7B). Immunohistochemistry showed phospho-Smads1, 5 and 8 (indicative of active Bmp signalling) were present in stage 10 cranial mesoderm, whereas Smad activity was lower when Shh signalling is initiated within the cranial mesoderm by HH stage 15 (Fig. 7D–G; data not shown). Likewise, Wnts are expressed in the cranial ectoderm and neural tube (Tzahor et al., 2003). This suggested that the Bmp and the canonical Wnt signalling pathways may antagonise Shh signalling in the early cranial mesoderm.

To investigate this hypothesis, beads pre-soaked in the Bmp antagonist Noggin or the Wnt antagonist Dkk1 (or Sfrp2) were

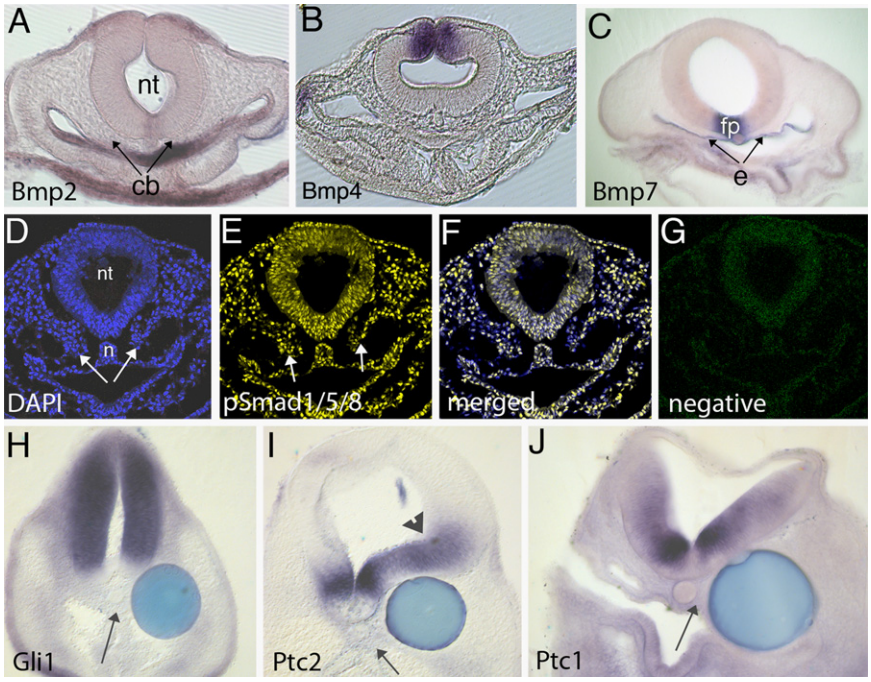

Fig. 7. Wnts and Bmps do not antagonise Shh signalling in the early cranial mesoderm. (A–C) and (H–J) are vibratome sections through the cranial base of HH stage 10 (A–C) and 13/14 (H–J) chick embryos showing the expression of (A) *Bmp2*, (B) *Bmp4*, (C) *Bmp7*, (H) *Gli1*, (I) *Ptc2* and (J) *Ptc1*. In (H–J) beads soaked in (H) Noggin, (I) Sfrp2 or (J) both Noggin and Sfrp2 have been placed into the cranial mesoderm of HH stage 10 embryos which were then allowed to develop for 24 h. (D–G) are immunolocalisation studies showing Smad1, 5 and 8 activity in the developing cranial base at HH stage 10 (D), DAPI stain, (E) Smad localisation and (F) the overlay of (D) and (E). (G) is the negative control. The arrows in (A, D, E and H–J) indicate the developing cranial base and in (C), the cranial endoderm. The arrowhead in (I) indicates induction of gene expression within the neural tube. cb, cranial base; e, endoderm; fp, floor plate; n, notochord; and nt, neural tube.

placed in the cranial mesoderm of HH stage 10 chick embryos at the level of the midbrain. Embryos were allowed to develop for 24 h until HH stage 13/14 when they were analysed by whole-mount *in situ* hybridisation for *Ptc1*, *Ptc2* and *Gli1* expression. None of these treatments resulted in premature or ectopic activation of *Ptc1* (Noggin 3/3, Dkk 2/2, Sfrp2 2/2), *Ptc2* (Noggin 4/4, Dkk 3/3, Sfrp2 5/5) or *Gli1* (Noggin 5/5, Dkk 2/2, Sfrp2 6/6) (Fig. 7H and I). Furthermore, simultaneous application of Noggin and Sfrp2 did not activate the Shh signalling pathway either (Fig. 7J; *Ptc1* 2/2, *Ptc2* 2/2, *Gli1* 2/2). As positive controls we confirmed the activity of the recombinant proteins by testing their ability to down-regulate *Wnt11* expression in the somite (Dkk1, Sfrp2) and *Msx1* expression in the developing facial primordia (Noggin, data not shown). We also determined the expression patterns of Gas1, Hip1 and Rab23, other potential antagonists of the Shh signalling pathway, and found no detectable expression in the cranial mesoderm (data not shown). Together, the data suggest that neither Bmp nor canonical Wnt signalling inhibits the Shh pathway in the early cranial mesoderm. Unlike chondrogenic initiation in the sclerotome, antagonism of Bmp signalling may not be required for induction of chondrogenesis within the developing cranial base.

The cranial and caudal notochord has similar ability to activate the shh signalling pathway

The absence of transcriptional targets of Shh signalling in the early cranial mesoderm may indicate that Shh expressed by the notochord is not secreted and/or modified appropriately or that the early cranial mesoderm is unresponsive to Shh signalling. To examine these possibilities, we tested the ability of the cranial *versus* caudal notochord to activate *Ptc/Gli1* expression when grafted into the trunk and cranial mesoderm respectively. We also examined the ability of the cranial notochord to activate Shh signalling in the neural crest derived mesenchyme in the head. Activation of the Shh signalling pathway was determined 24 h later by whole-mount *in situ* hybridisation for the transcriptional targets of Shh: *Ptc1*, *Ptc2* and *Gli1*.

Grafting of the posterior trunk notochord into the head activated the Shh signalling pathway in the lateral neural-crest derived mesenchyme and ectopically within the neural tube (Fig. 8A–C; *Ptc1* 2/2, *Ptc2* 2/2, *Gli1* 4/4). However, within the presumptive cranial base, the ectopic trunk notochord did not strongly activate or significantly increase Shh signalling. This is dramatically illustrated in Fig. 8C where the ectopic notochord is in close proximity to the developing cranial base. Ectopic *Gli1* transcription was strongly activated in the lateral cranial neural-crest derived mesenchyme and neural tube, but there was no detectable *Gli1* expression in the developing cranial base either around the endogenous notochord or adjacent to the ectopic notochord. In the embryos shown in Fig. 8A and B there was a slight increase in the expression of the transcriptional targets of Hh signalling within the cranial mesoderm on the side with the ectopic notochord. However, the levels of *Ptc1*, *Ptc2* and *Gli1* expression were much lower than those induced in the neural crest-derived mesenchyme (arrowed), suggesting that the cranial mesoderm is in fact relatively refractory to Hh signalling.

This conclusion was further supported by the grafts of a cranial notochord into the trunk that strongly induced the expression of Shh transcriptional targets within the paraxial mesoderm (Fig. 8D and E; *Ptc1* 1/1, *Ptc2* 2/2). Likewise, grafts of a cranial notochord ectopically within the head were able to activate Shh signalling in surrounding tissues although again activation of Shh signalling targets was higher lateral to the ectopic notochord *i.e.* in the neural crest derived mesenchyme (Fig. 8F; $n=4/4$). This rules out the possibility that Shh is not secreted from the cranial notochord, for example, due to the absence of Dispatched necessary for Shh secretion. The combined data show that the cranial notochord can activate Shh signalling and that the early cranial mesoderm is refractory to Shh signalling.

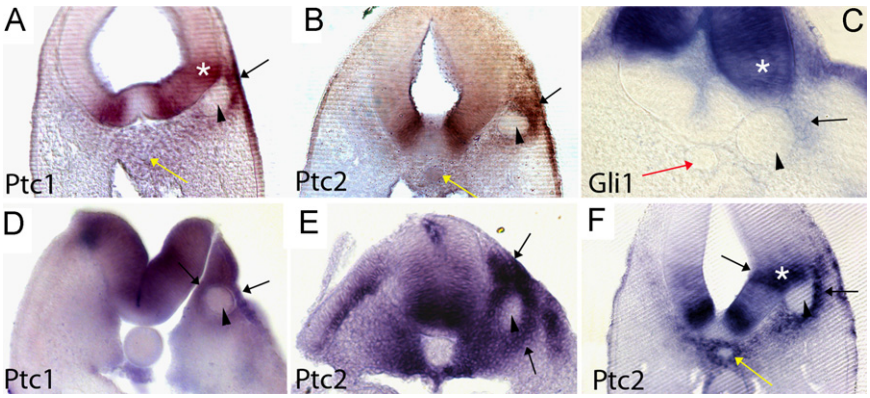

Fig. 8. The cranial mesoderm is refractory to Shh signalling. Transverse vibratome sections through the presumptive cranial base (A–C and F) or somite (D and E) of HH stage 13/14 (A–E) and HH stage 15 (F) chick embryos which have been probed for the expression of *Ptc1* (A and D), *Ptc2* (B, E and F) and *Gli1* (C). In (A–C) the trunk notochord has been grafted into the head whereas in (D–F), the cranial notochord has been grafted into either the trunk (D and E) or head (F). The arrowheads indicate the ectopic notochord. The black arrows indicate the induction of gene expression and yellow or red arrows indicate the position of the original notochord. ⁎indicates ectopic expression within the neural tube.

Discussion

It has been generally assumed that the molecular mechanisms required for development of the vertebrae and posterior cranial base, are conserved. Both arise from paraxial mesoderm in close proximity to the notochord and floor plate and the development of both requires the function of the homeobox gene, Bapx1 (Lettice et al., 1999; Tribioli et al., 1997). The somitic contribution to the occipital bone has strengthened this assumption as the occipital bone is a modified vertebral element that has expanded to support the brain (Couly et al., 1992, 1993; Evans and Noden, 2006; Muller and O'Rahilly, 1994; Kuratani, 2005). However, as stated by Evans and Noden (2006), the craniofacial mesoderm is a unique region which is exposed to distinct tissue interactions from the neural crest and is in close proximity to the endoderm; so, it cannot be assumed the interactions will be the same. Building on data by Nie et al., 2005, which showed that Shh and Sox9 are expressed in adjacent tissues at the time of cranial base induction, we have examined these assumptions comparing and contrasting the development of the cranial *versus* axial skeleton in developing chick and mouse embryos. We show that a distinct molecular repertoire controls cranial base development: Pax1, a homeobox gene required for chondrogenesis in the trunk, is not expressed in the unsegmented cranial mesoderm and unlike in the trunk, *Bapx1* is expressed prior to activation of the Shh signalling pathway. Also, in contrast to the trunk, cranial base induction does not appear to require antagonism of Bmp signalling. However, as in the trunk, Shh is required for the induction of chondrogenesis. Finally, we show that development of the cranial base occurs much later than the axial skeleton and this difference in the timing of cranial base *versus* axial skeleton development occurs because the cranial mesoderm is refractory to Shh signalling.

The onset of chondrogenic differentiation in the cranial base coincides with the activation of Shh signalling and in the absence of Hh signalling we did not detect any chondrogenic differentiation within the posterior cranial base. We propose that Hh signalling is required for the initiation of chondrogenesis within the developing cranial base. We have also shown that Shh signalling within the cranial base is required for cell survival as has been shown in several tissues, including the developing sclerotome, neural tube, limb bud, prechordal plate and cranial neural crest cells (Ahlgren and Bronner-Fraser, 1999; Borycki et al., 1999; Chiang et al., 1996; Aoto et al., 2009). The decrease in cell survival in Shh deficient embryos may result in the absence of sufficient chondrogenic progenitors for cranial base development. However, in contrast to the neural tube, forebrain, face and limb, cell death does not appear to be the result of ectopic repressor forms of Gli3 in the absence of Shh as cranial base development is not rescued in Shh/Gli3 double mouse mutants (Aoto et al., 2002, 2009; Litingtung and Chiang, 2000; Litingtung et al., 2002; te Welscher et al., 2002).

Our analysis of the onset of chondrogenic induction reveals that cranial base development is initiated later than the axial skeleton. As we did not observe qualitative differences in the ability of cranial and caudal notochord to active Shh signalling, it is curious that there should be this temporal difference in cranial base development relative to the trunk axial skeleton. We hypothesize that this relative delay is necessary for the rapid growth of the developing brain and may also facilitate the co-ordination of the formation of the anterior and posterior cranial base that develop from the neural crest and mesoderm respectively. In the chick, neural crest cells start to migrate around HH stages 9/10 and do not reach their destination until 24 h later at approximately HH stage 13/14. At HH stage 10, axial skeletal, but not posterior cranial base, development has already been initiated. Posterior cranial base development does not begin until HH stage 17 just after the arrival of the neural crest cells into the anterior region of the head. The relative delay in cranial base development may also co-ordinate development of the cranial base with the neural-crest derived skeletal structures that form within the pharyngeal arches.

Our studies clearly indicate that the cranial notochord secretes functionally active Shh and are consistent with the role of Shh in the notochord and floor plate during the specification of the ventral otocyst which lies adjacent to the developing cranial base (Riccomagno et al., 2002). Our data show that the cranial mesoderm is initially refractory to Hh signalling. The molecular basis for this regulation remains obscure. This is not simply due to the absence of the Smoothened receptor, which was detected by RT-PCR (data not shown) or the presence of known antagonists of the Shh signalling pathway. As the acrochordal cartilage arises first at the tip of the notochord and adjacent to Rathke's pouch—both sources of Shh signalling—we consider an additional possibility is that quantitative differences in the levels of Shh signalling not detectable with our notochord graft studies exist. This possibility would be consistent with the timing of the onset of cranial base development when Shh is expressed in the endoderm adjacent to the developing cranial base. Indeed, Shh is not expressed in the endoderm at HH stage 14 prior to the initiation of chondrogenesis within the cranial base but transcripts are detectable at stage 17 (compare Supplementary Fig. 2B and C).

Cranial base induction also follows the migration of neural crest into the forming face and pharyngeal arches. This raised the possibility that as for cranial myogenic induction, invading neural crest cells may be required to insulate the presumptive cranial base from inhibitory signals from the ectoderm and endoderm (Tzahor et al., 2003). We hypothesised that Bmp and canonical Wnt signalling pathways could potentially antagonise Shh signalling in the early cranial mesoderm and that these repressive signals would be counteracted by the migration of neural crest cells which express Bmp and Wnt antagonists (Ladher et al., 2000; Tzahor et al., 2003). If this were true it would also couple the induction of cranial myogenesis with that of the cranial base. However, application of the Bmp antagonist, Noggin, or the Wnt antagonists, Sfrp2 or Dkk1, failed to activate the Shh pathway demonstrating that the induction of myogenic and chondrogenic differentiation are molecularly uncoupled. Furthermore, this demonstrates that unlike in the trunk, antagonism of Bmp signalling may not be required for chondrogenic commitment in the cranial base anlagen. Other candidate antagonists include retinoic acid signalling. However, retinoic acid signaling varies throughout the head with high levels in the somites (due to Raldh2 expression) and very low levels of retinoic acid activity in the unsegmented cranial mesoderm (due to Cyp26 expression) (Swindell et al., 1999; Reijntjes et al., 2004). There are graded levels of retinoic acid activity in the mesoderm adjacent to the hindbrain. As such, there is no correlation with retinoic acid and the absence of Shh signaling in the developing occipital somites and unsegmented cranial paraxial mesoderm. We, therefore, hypothesize that retinoic acid is unlikely to modulate Shh signaling throughout the cranial mesoderm. Although we favour the hypothesis that factors expressed by neural crest cells permit the onset of chondrogenesis, the expression of chondrogenic markers within the notochord and neural crest cells prevents us from directly testing this at present, for example by culture of the presumptive cranial mesoderm and notochord with neural crest cells or conditioned media derived from neural crest cells.

In the cranial base, following commitment to the chondrogenic lineage there is a prolonged delay in the onset of terminal differentiation marked by collagen type X expression (Koyama et al., 2007). This temporal protraction between commitment and terminal differentiation has previously been observed for the developing cranial musculature (Noden et al., 1999) and presumably allows rapid growth of the musculoskeletal system whilst the head is undergoing considerable expansion and morphogenetic change. Terminal differentiation would only occur when morphogenesis is complete and all the structures have been established.

Finally we also show that the hedgehog pathway is not activated during the initial stages of development of the sensory capsules, the otic, nasal and optic cartilages, in the developing chick embryo. Supporting our findings in chicks, we observed *Sox9* expression in the developing otic capsules of *Shh*$^{-/-}$ mutant embryos. These observations are consistent with data showing that Shh is not sufficient to initiate otic capsule development and TGFβ/FGF2 can induce development of the otic capsule (Frenz et al., 1994; Liu et al., 2002). Furthermore, the nasal septum and otic capsules are present at E18.5 in *Shh*$^{-/-}$ mutant mice (Aoto et al., 2009). FGF, but not Hh, signalling has also been implicated in the induction of chondrogenesis within cranial neural crest cells of the developing face (Abzhanov and Tabin, 2004; Sarkar et al., 2001). Therefore, we conclude that Shh is required for the induction of the midline skeletal structures of the posterior cranial base but acts through a signalling network that is distinct from that in the trunk. The precise timing of cranial base development may also vary between species being dependent on the balance of Hedgehog signals from the floor plate, notochord, cranial ectoderm, endoderm and Hh antagonists. We also propose that distinct molecular mechanisms have evolved for the induction of the cartilages of the cranial base, pharyngeal arches and sensory capsules.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.08.011.

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